FEBS Letters 585 (2011) 2951–2957

FEBS Letters

journal homepage: www.FEBSLetters.org

Signal-peptide-peptidase-like 2a (SPPL2a) is targeted to lysosomes/late endosomes by a tyrosine motif in its C-terminal tail

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ARTICLE INFO

Article history:
Received 11 May 2011
Revised 29 August 2011
Accepted 29 August 2011
Available online 2 September 2011

Edited by Lukas Huber

Keywords:
Regulated intramembrane proteolysis
SPPL2a
Lysosome
Lysosomal targeting
Tyrosine-based sorting motif

ABSTRACT

Signal-peptide-peptidase-like 2A (SPPL2a), an aspartyl intramembrane protease, has been implicated in the proteolysis of TNF-alpha, Fas Ligand and Bri2. Here, we show that endogenous SPPL2a – in agreement with overexpression studies – is localised in membranes of lysosomes/late endosomes. Furthermore, we have analysed the molecular determinants for lysosomal sorting of SPPL2a by creating chimaeric constructs between SPPL2a and its plasma membrane localised homologue SPPL2b. Lysosomal transport of SPPL2a critically depends on its cytosolic carboxyterminal tail. A canonical tyrosine-based sorting motif of the YXXø type at position 498 is sufficient to direct SPPL2a to lysosomal/late endosomal compartments. This motif accounts for the differential localisation of the homologous proteases SPPL2a and SPPL2b and thereby influences the access to substrates and biological function of SPPL2a.

Structured summary of protein interactions:
LAMP2 and **SPPL2a** colocalize by fluorescence microscopy (view interaction)

1. Introduction

Regulated intramembrane proteolysis (RIP) refers to a two-step proteolytic cascade comprising the ectodomain release from a transmembrane substrate followed by an intramembrane cleavage and liberation of an intracellular domain [1]. RIP has emerged over the last years as an important principle in cellular signaling [1,2].

Signal-peptide-peptidase (SPP) and its homologues Signal-peptide-peptidase-like (SPPL) 2a, -2b, -2c, and -3 belong to the class of aspartyl intramembrane-cleaving proteases (I-CliPs). They show a substrate preference towards type 2 transmembrane proteins [2]. Within the SPP/SPPL family, a functional relationship between SPPL2a and SPPL2b was suggested [3].

SPPL2a and/or SPPL2b have been implicated in the intramembrane cleavage of TNF-α [3], Bri2 [4] and Fas ligand [5]. SPPL2a and -b are 50% identical and 70% homologous to each other [2]. In mRNA analyses, SPPL2a was found to be expressed in all major human adult tissues, whereas SPPL2b transcripts were primarily detected in the adrenal cortex and the mammary gland [6].

SPPL2a and SPPL2b exhibit distinct subcellular localisations. Overexpressed SPPL2a was observed in late endosomal compartments colocalising with Rab-7 [3]. In agreement, endogenous SPPL2a was found to be enriched in lysosomal membranes isolated from human placenta in an organellar proteomic study [7]. In contrast, overexpressed SPPL2b has been detected at the plasma membrane [3].

It seems likely that the divergent subcellular localization in addition to the distinct tissue expression pattern of the two proteases accounts for individual substrate preferences. Subcellular trafficking of proteases as well as of substrates is a well-recognised mechanism of regulating RIP [8]. For targeting to lysosomes, membrane proteins depend on distinct short motifs contained within their cytoplasmic domains. These are able to interact with adaptor protein complexes in the cytosol [9]. Depending on the critical residues, canonical lysosomal targeting motifs are classified as tyrosine- or dileucine-based signals [9].

We have analysed the molecular requirements for lysosomal/late endosomal sorting of SPPL2a in comparison to the

Abbreviations: RIP, regulated intramembrane proteolysis; SPP, signal-peptide-peptidase; SPPL, signal-peptide-peptidase-like; FasL, Fas ligand; TNF-α, tumor necrosis factor α; BMP, bis(monoacylglycero)phosphate; GAPDH, glyceraldehyde-3-phosphate-dehygrogenase; MEF, murine embryonic fibroblast

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doi:10.1016/j.febslet.2011.08.043

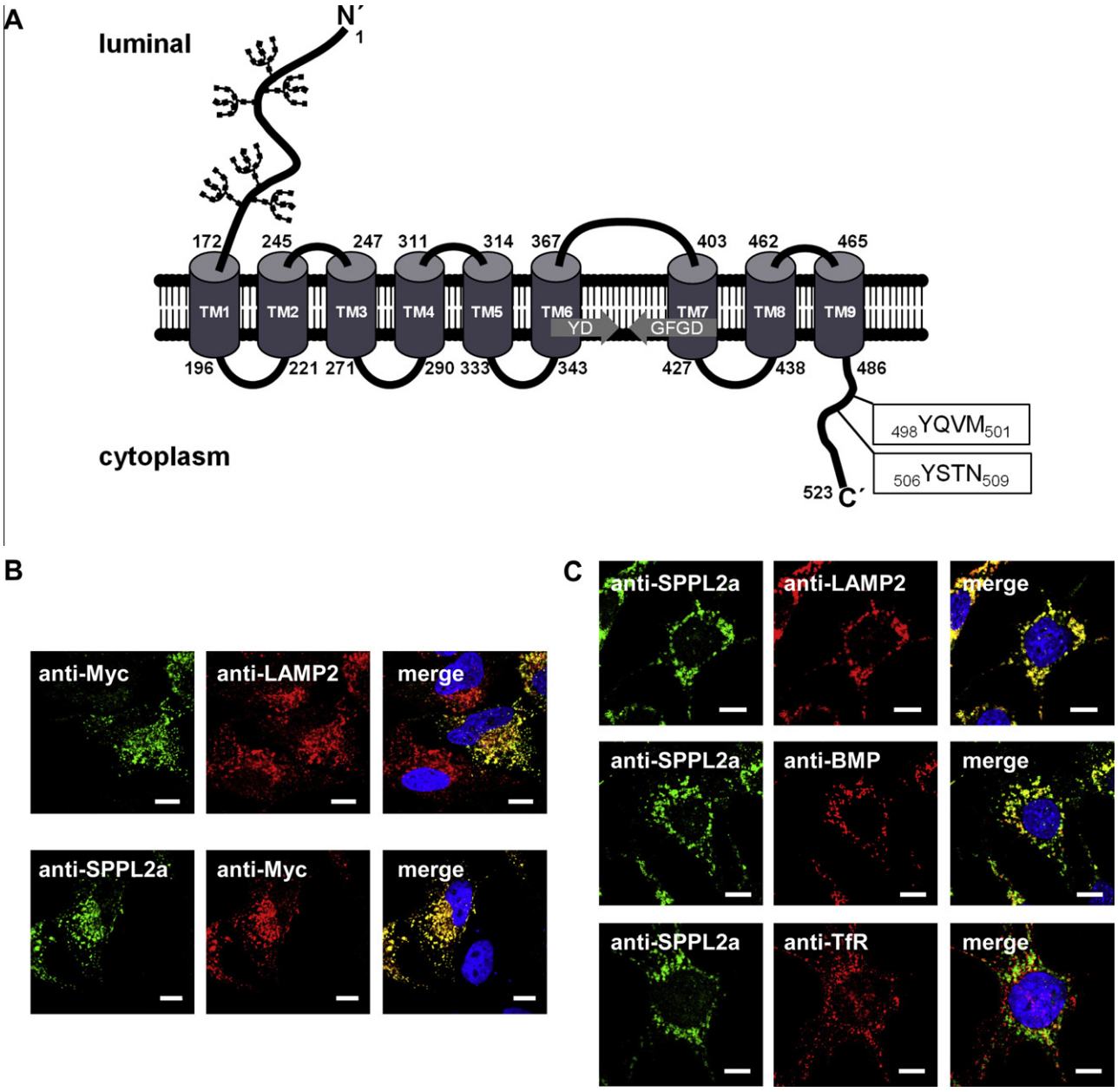

Fig. 1. Predicted topology and lysosomal/late endosomal localisation of SPPL2a. (A) The topology of murine SPPL2a as predicted by TMHMM (http://www.expasy.org). Numbers indicate the limiting extra-membraneous residues. Positions of putative tyrosine-based lysosomal targeting motifs, N-glycosylation sites and the catalytically critical YD and GFGD motifs are marked. (B) HeLa cells were transiently transfected with SPPL2a-Myc. Overexpressed SPPL2a was visualised using anti-Myc (upper and lower panel) or a novel antibody against the native SPPL2a protein (lower panel). In the upper panel lysosomes/late endosomes were revealed with an antibody against LAMP2. (C) In MEF cells endogenous SPPL2a was detected with the novel antibody validated above (B, lower panel). Co-staining with antibodies against LAMP2 and BMP (lysosomes/late endosomes) and the transferrin receptor (TfR, early and recycling endosomes) was performed. Scale bars: 10 μm.

plasma-membrane localised homologue SPPL2b. Using a strategy based on SPPL2a/b chimaeric constructs and mutagenesis of putative targeting motifs within SPPL2a, we could demonstrate that a single tyrosine-based sorting motif within SPPL2a is sufficient to mediate transport of this protease to lysosomes.

2. Materials and methods

2.1. Antibodies

Antibodies against the myc epitope were purchased from Cell signaling (Danvers, MA, USA) and Santa Cruz (Santa Cruz, CA, USA). A monoclonal antibody against h-LAMP2 (2D5) has been described previously [10] and anti-BMP (*bis*(monoacylglycero) phosphate, 6C4) was kindly provided by Jean Gruenberg, University of Geneva. Anti-transferrin receptor (Clone H68.4) and anti-glyceraldehyde-3-phosphate-dehygrogenase (GAPDH) were obtained from Invitrogen (Carlsbad, CA, USA) and Sigma–Aldrich (Taufkirchen, Germany). Secondary antibodies were purchased from Molecular probes (Eugene, OR, USA) and Dianova (Hamburg, Germany).

An antiserum against the native SPPL2a protein was produced by cDNA immunisation. Expression constructs were generated by cloning the murine and human SPPL2a ORFs into the pcDNA3.1/Hygro+ vector (Invitrogen, Carlsbad, CA) and conjugated to 1-μm gold particles (Bio-Rad, Hercules, CA, USA). Rabbits were immunised by ballistic DNA immunization with a mixture of both

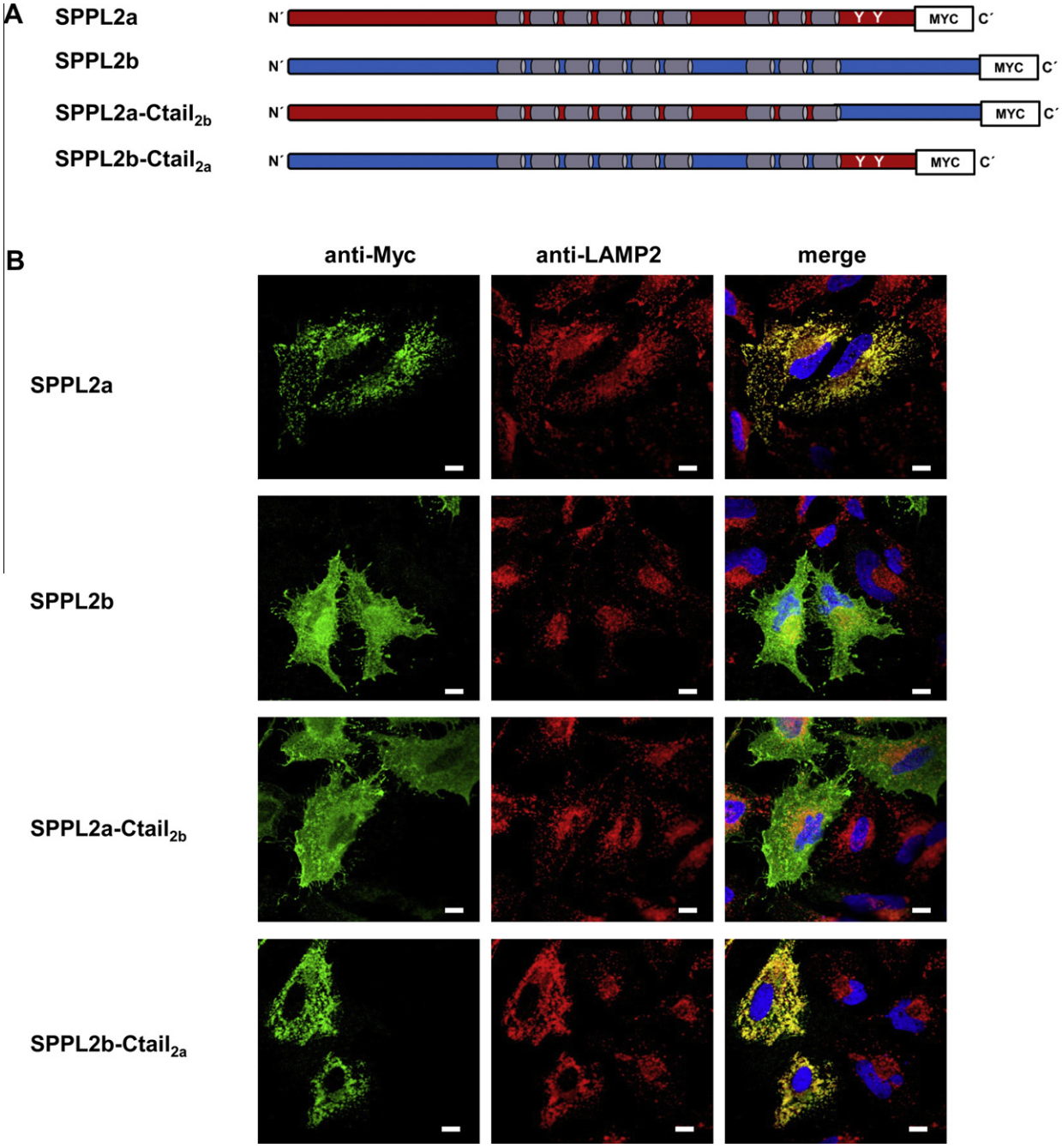

Fig. 2. The carboxyterminal cytosolic domain of SPPL2a mediates lysosomal/late endosomal targeting. (A) Schematic representation of SPPL2a (red) and SPPL2b (blue) wild type as well as SPPL2a/b chimaeric proteins (SPPL2a-Ctail$_{2b}$, SPPL2b-Ctail$_{2a}$). (B) HeLa cells were transiently transfected as indicated and the distribution of the respective proteins was analysed by indirect immunofluorescence using anti-Myc. Lysosomes/late endosomes were visualised with anti-LAMP2. Scale bars: 10 μm.

expression plasmids. Animals received four immunizations. A serum sample was obtained 10 days after the last immunisation [11]. The complete serum was used for immunocytochemical stainings at a 1:100 dilution. For Western blot detection of murine SPPL2a, an antiserum was generated against a synthetic peptide (residues 77–94, CHLSDIPPDGIRNKAVVVH). After affinity purification against the immobilised peptide the antibody was employed at a final concentration of 6 μg/ml for Western blotting.

2.2. Cloning of SPPL expression constructs, SPPL2a mutants and SPPL2a/b chimaera

The ORFs of murine SPPL2a and SPPL2b were fused to a Myc tag at their 3′ end by PCR and subcloned into pcDNA3.1/Hygro+. Mutations of putative targeting signals within the murine SPPL2a ORF (Fig. 3A) were introduced by overlap-extension PCR. For the generation of the chimaeric constructs (Figs. 2A and 4A), fragments corresponding to amino acid residues 1–485 of murine SPPL2a and residues 1–484 of murine SPPL2b were fused to PCR products corresponding to amino acids 485–578 of SPPL2b and 486–523 of SPPL2a, respectively. All chimaeric constructs contained a 3′-Myc tag.

2.3. Cell culture and transfection

Murine embryonic fibroblasts (MEF) and HeLa cells were maintained in DMEM (Dulbecco's modified Eagle's medium; PAA, Cölbe, Germany) supplemented with 10% (v/v) FBS (fetal bovine serum;

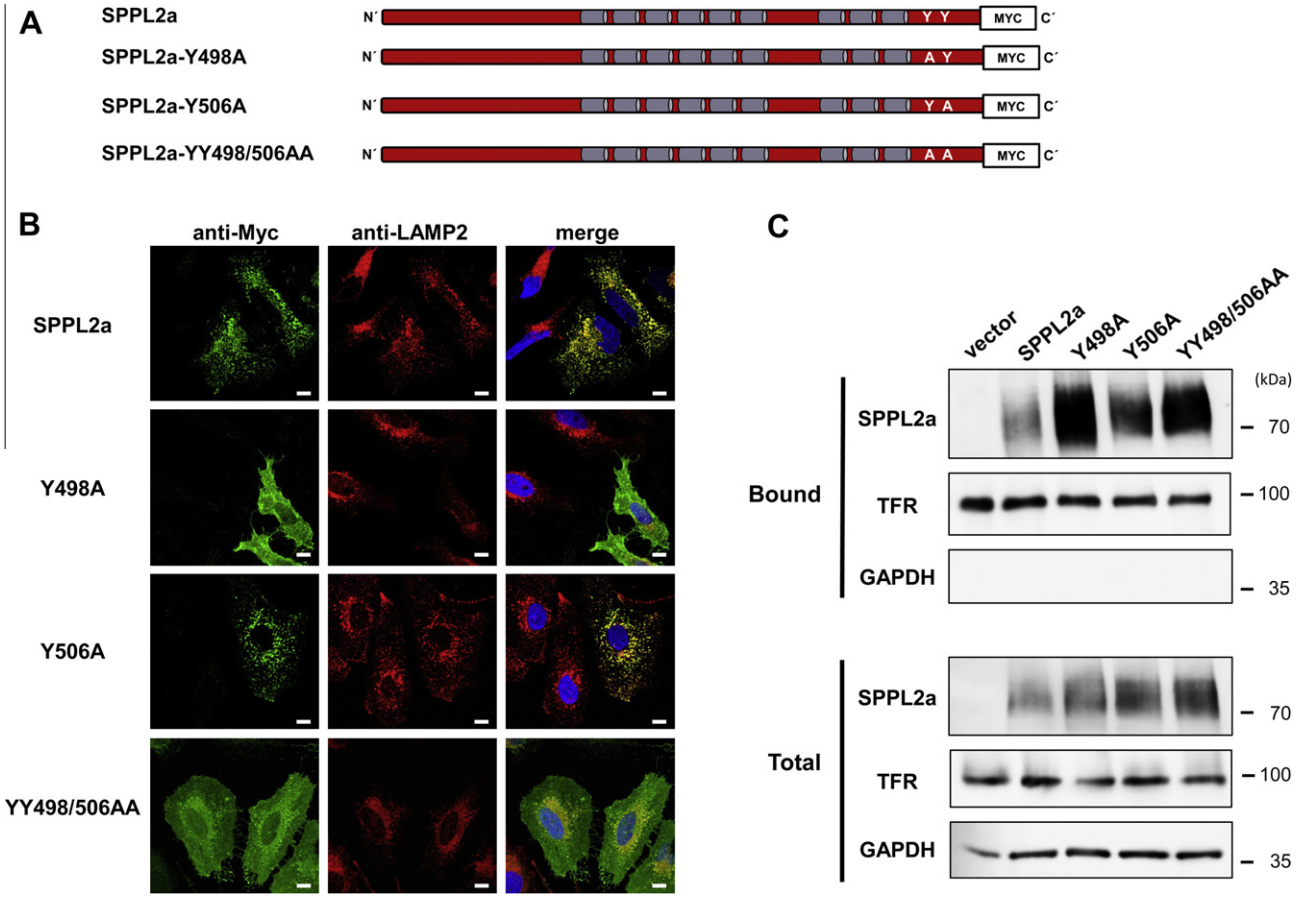

Fig. 3. A single tyrosine-based targeting motif is critical for lysosomal/late endosomal targeting of SPPL2a. (A) Scheme of SPPL2a illustrating positions of the two tyrosine residues that were mutated. (B) Hela cells were transiently transfected with wild type or mutant SPPL2a as indicated and the overexpressed protein detected with anti-Myc. Lysosomes/late endosomes were visualised in parallel by co-staining against LAMP2. Scale bars: 10 μm. (C) Surface biotinylation of HeLa cells transiently transfected with empty vector or SPPL2a, wild-type or mutant, as indicated. Aliquots of total lysates (430 μg protein) were used for pulldown with streptavidin agarose. After washing, biotinylated proteins (Bound) were recovered in 100 μl SDS–PAGE sample buffer. For Western blotting, 35 μl of the eluted biotinylated proteins (Bound) and total lysate aliquots (15 μg protein) were subjected to SDS–PAGE meaning, that, in relative terms, the analysed sample of streptavidin-bound proteins was derived from a 10-fold higher amount of material. Signals shown for "Bound" and "Total" samples were derived from the same blotting membrane and exposure. Membranes were probed with antibodies detecting SPPL2a, the transferrin receptor (TFR, positive control) or the cytosolic protein GAPDH as control for the specificity of the biotinylation and equal protein loading, respectively.

PAA), 100 units/ml penicillin (PAA) and 100 μg/ml streptomycin (PAA). Cells were seeded 24 h prior to transfection and transfected with Turbofect (Fermentas, St. Leon-Rot, Germany) as described previously [12].

2.4. Indirect immunofluorescence

Immunocytochemical stainings were performed as described previously [12]. Cells were fixed 48 h after transfection. Photographs of optical sections were acquired with an Olympus FV1000 confocal laser scanning microscope.

2.5. Surface biotinylation and streptavidin pulldown of biotinylated proteins

Cell surface proteins were labelled 48 h after transfection with the membrane impermeable, cleavable reagent sulfo-NHS-SS-biotin [sulfosuccinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate] (Thermo Scientific, Waltham, MA, USA) as described before [13]. Cells were harvested, lysates prepared as described [12] and protein concentration determined with a BCA protein assay kit (bicinchoninic acid, Thermo scientific). Aliquots of lysates (430 μg protein) were subjected to pulldown with 50 μl of high-capacity streptavidin agarose resin (Thermo Scientific) [13]. Biotinylated proteins were eluted from the beads in 100 μl reducing SDS/PAGE sample buffer. For Western blot analysis [12], 35 μl of the eluate were subjected to SDS–PAGE [14]. Aliquots from total cell lysates (15 μg of protein) were analysed in parallel. Bands were quantified by densitometry and the percentage of surface-localised SPPL2a was calculated (mean of 2 independent experiments).

3. Results

3.1. SPPL2a is a lysosomal/late endosomal membrane protein

We could show that overexpressed myc-tagged murine SPPL2a in HeLa cells is present in lysosomes/late endosomes as revealed by colocalisation with the lysosomal/late endosomal membrane protein LAMP2 (Fig. 1B, upper panel). A newly generated antibody against native SPPL2a also recognised the overexpressed protein in HeLa cells (Fig. 1B, lower panel). In murine embryonic fibroblasts, this antibody was able to visualise endogenous SPPL2a which colocalised with LAMP2 as well as the lysosomal/late endosomal lipid BMP (Fig. 1C). No colocalisation with early and recycling endosomes (transferrin receptor) was observed (Fig. 1C).

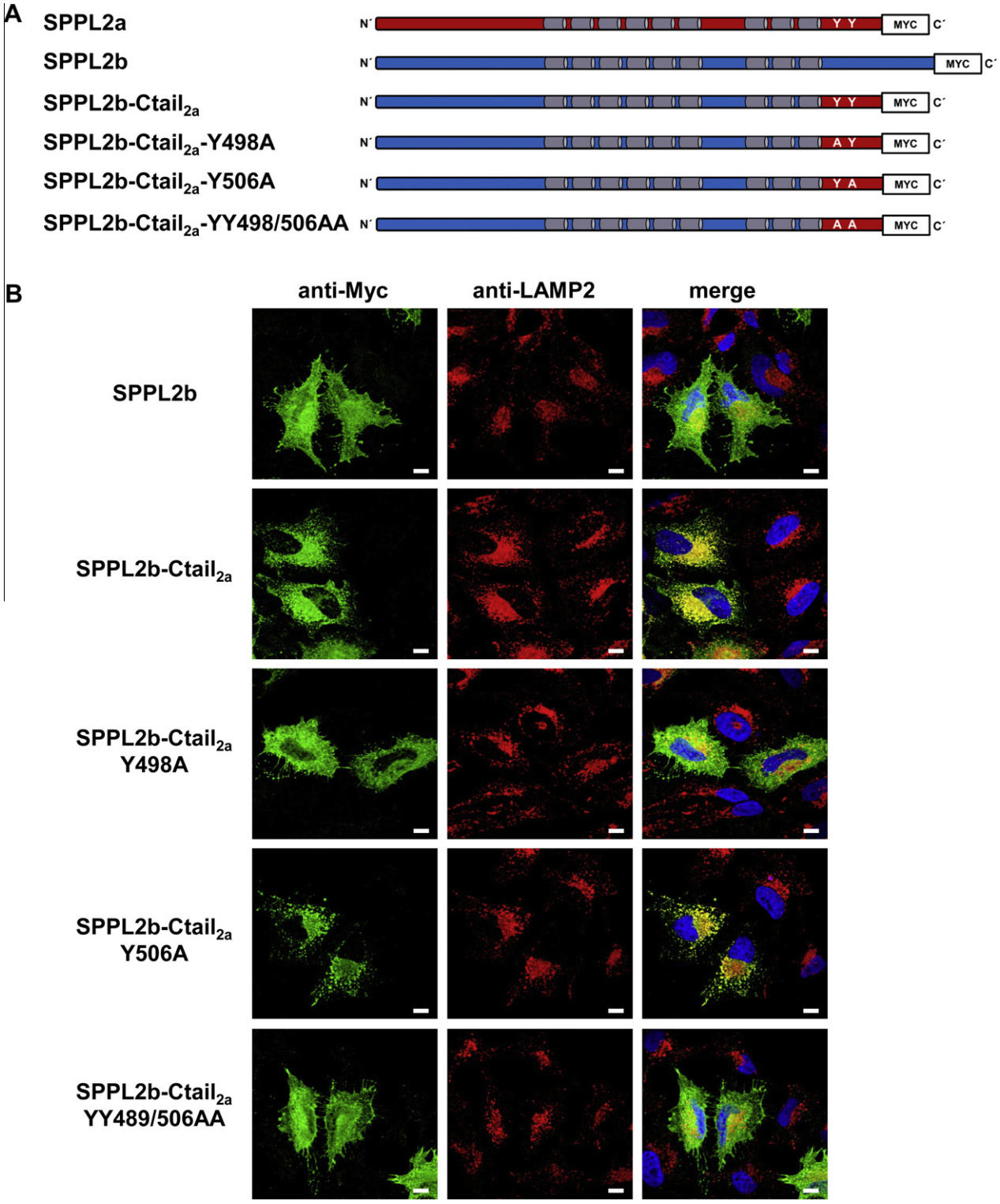

Fig. 4. Y498 is the critical lysosomal/late endosomal sorting determinant in the cytosolic tail of SPPL2a. (A) Scheme of SPPL2b-Ctail$_{2a}$ chimaeric proteins including introduced mutations. (B) HeLa cells transiently expressing SPPL2b wild type or SPPL2b-Ctail$_{2a}$ chimaeric proteins were analysed by indirect immunofluorescence utilising anti-Myc. For co-localisation analysis, co-staining with anti-LAMP2 was performed. Scale bars: 10 μm.

This shows that also endogenous SPPL2a is a constituent of lysosomal/late endosomal membranes. In contrast, overexpressed SPPL2b was detected mainly at the plasma membrane (Fig. 2B).

The currently assumed topology of SPPL2a is characterised by a luminal glycosylated N-terminal domain, nine transmembrane segments and a short C-terminal cytoplasmic tail (Fig. 1A). Within the 38 residues comprising C-terminal domain of SPPL2a two motifs matching the consensus sequences of established tyrosine-based lysosomal/endosomal targeting signals are present (Fig. 1A): YQVM (residues 498–501) and YSTN (residues 506–509). In SPPL2b no lysosomal targeting sequence was detected (not shown).

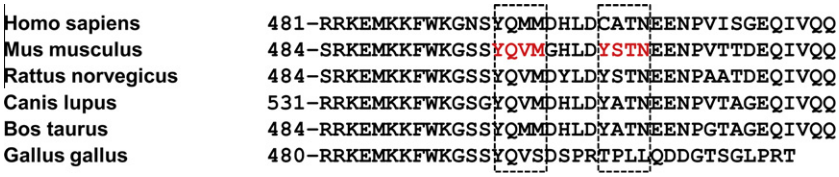

Fig. 5. Multispecies alignment of the cytosolic C-terminal domain of SPPL2a. The putative sorting motifs around Y498 and Y506 are shown in red in the murine sequence and marked by dashed boxes.

3.2. The carboxyterminal tail of SPPL2a is needed for lysosomal transport

The differential localisation of both proteases prompted us to analyse the relevance of the C-terminal domain of SPPL2a for lysosomal targeting by generating chimaeric proteins (Fig. 2A). Replacement of the cytosolic domain of SPPL2a by that of SPPL2b in the SPPL2a-Ctail$_{2b}$ chimaera resulted in a loss of lysosomal/late endosomal targeting and delivery of the protein to the plasma membrane in HeLa cells (Fig. 2B). On the contrary, the C-terminal tail of SPPL2a was able to redirect SPPL2b from the cell surface to intracellular compartments, mostly representing lysosomes/late endosomes (Fig. 2B). These results show that the cytosolic tail of SPPL2a is necessary and sufficient for inducing lysosomal targeting.

3.3. The proximal tyrosine motif in the cytosolic tail of SPPL2a is required for lysosomal sorting

We investigated a possible contribution of the two putative tyrosine-based sorting motifs at Y498 and Y506 by site-directed mutagenesis (Fig. 3A) and overexpression of the mutants in HeLa cells (Fig. 3B). Mutation of the proximal tyrosine residue (Y498A) impaired lysosomal/late endosomal targeting of SPPL2a and significant amounts of the mutant protein were detected at the plasma membrane. In contrast, mutation of the distal motif (Y506A) had no influence on the localisation of SPPL2a. Like the single Y498A mutant, a double mutant of both motifs (YY498/506AA) was observed at the plasma membrane to a predominant degree (Fig. 3B).

In an alternative approach, the surface expression of wild type SPPL2a and the tyrosine mutants was analysed biochemically by surface biotinylation of transfected HeLa cells with a membrane impermeable reagent (Fig. 3C). Interestingly, significant, but minor amounts of wild type SPPL2a were recovered in the "Bound" fraction indicating plasma membrane localization of the overexpressed protein which had not been detected by immunofluorescence. However, the level of surface expression (% of total protein) of the Y498A (44%) and the YY498/506AA double mutant (32%) was considerably higher than that of wild-type SPPL2a (17%) and the Y506A mutant (16%).

These results argue for a single functional lysosomal sorting motif in the cytosolic tail of SPPL2a at Y498. As a final proof, we went back to the system of the SPPL2a/b chimaera (Figs. 2 and 4) and introduced the mentioned tyrosine mutations in the SPPL2b-Ctail$_{2a}$ chimaera (Fig. 4A) which was delivered to a significant degree to lysosomes/late endosomes (Fig. 4). Similar to wild type SPPL2a, introduction of the Y498A mutation into this chimaera abolished lysosomal/late endosomal targeting and restored plasma membrane localisation, which was not achieved by the Y506A mutant. As expected the combination of both mutations also resulted in localisation at the cell surface.

4. Discussion

In the present study we have revealed that lysosomal/late endosomal sorting of murine SPPL2a depends on a single tyrosine-based motif (Y498) in its cytoplasmic tail. In a multispecies alignment of SPPL2a (Fig. 5), a high degree of conservation of this motif becomes evident. The identification of a functional sorting motif within the C-terminal domain provides indirect proof that the C-terminus of SPPL2a is localised in the cytosol since cytoplasmic exposure is a prerequisite for functionality of such a signal.

The identified motif $_{498}$YQVM$_{501}$ fully corresponds to the YXXø consensus sequence of tyrosine-based lysosomal sorting motifs [9]. Within these types of signals, the Y residue is essential for functionality and recruitment of the adaptor protein complexes AP-1 and AP-2 [9]. Ø represents an amino acid with a bulky hydrophobic side chain, which may be V, L, I, F or M [9]. A frequent feature of many functional tyrosine-based sorting motifs is a glycine residue directly preceding the tyrosine which was shown to modulate the strength of the motif [9]. Such a glycine residue is apparently missing in most of the SPPL2a sequences (Fig. 5), only SPPL2a in *Canis lupus* exhibits one. Commonly, lysosomal YXXø signals are localised within six to nine residues to the adjacent transmembrane segment. In the case of murine SPPL2a, twelve amino acids separate the tyrosine Y498 from the last transmembrane segment, which results in a slightly wider distance to the membrane. In contrast to SPPL2a, several multispanning lysosomal membrane proteins, e.g. CLN3, mucolipin-1, LAPTM4α and TMEM192, exhibit multiple targeting motifs, that are often able to compensate for each other [13,15].

The observed degree of surface localisation for wild type SPPL2a (16%) is considerably higher than that reported for the lysosomal protein LAMP1 (2–3%) [16]. However, in the current set-up it cannot be excluded that overexpression has led to a saturation of the intracellular targeting machinery. In addition to direct trafficking routes, lysosomal membrane proteins, as exemplified by LAP (lysosomal acid phosphatase), can also follow an indirect sorting pathway via the plasma membrane [15]. Potentially, SPPL2a also utilises this pathway resulting in a transient association of SPPL2a with the plasma membrane. However, it is also conceivable that a small pool of SPPL2a is constitutively present at the cell surface. Further studies will be needed to clarify these issues, especially for the endogenous protein.

Functional data on SPPL2a and SPPL2b are very limited since only three substrates have been identified to date: TNF-α [3], Bri2 [4] and FasL [5]. Based on the widespread tissue expression of SPPL2a [6] it may be speculated that additional substrates remain to be identified or that SPPL2a fulfils a more generalised function in the lysosomal system. It has been reasoned that SPPL proteases – presumably in collaboration with the γ-secretase complex – may provide a system for disposing of membrane stubs arising from limited proteolytic cleavage events of transmembrane precursors and that these proteases in general contribute to the degradation of integral membrane proteins [1,2]. It can be anticipated that the identification of the lysosomal sorting determinants of SPPL2a achieved in this study will help to dissect the interconnections between function of the protease and its substrates on one hand and their subcellular localisation and trafficking on the other.

Acknowledgements

The authors thank Ruben Rose and Sebastian Held, Kiel, as well as Fabienne Seyfried and Gudrun Dubberke, Hamburg, for excellent technical assistance. This work was supported by the Deutsche Forschungsgemeinschaft as part of the SFB 877.

References

[1] Wolfe, M.S. and Kopan, R. (2004) Intramembrane proteolysis: theme and variations. Science 305, 1119–1123.
[2] Golde, T.E., Wolfe, M.S. and Greenbaum, D.C. (2009) Signal peptide peptidases: a family of intramembrane-cleaving proteases that cleave type 2 transmembrane proteins. Semin. Cell Dev. Biol. 20, 225–230.
[3] Friedmann, E., Hauben, E., Maylandt, K., Schleeger, S., Vreugde, S., Lichtenthaler, S.F., Kuhn, P.H., Stauffer, D., Rovelli, G. and Martoglio, B. (2006) SPPL2a and SPPL2b promote intramembrane proteolysis of TNFalpha in activated dendritic cells to trigger IL-12 production. Nat. Cell Biol. 8, 843–848.
[4] Martin, L., Fluhrer, R., Reiss, K., Kremmer, E., Saftig, P. and Haass, C. (2008) Regulated intramembrane proteolysis of Bri2 (Itm2b) by ADAM10 and SPPL2a/SPPL2b. J. Biol. Chem. 283, 1644–1652.
[5] Kirkin, V., Cahuzac, N., Guardiola-Serrano, F., Huault, S., Luckerath, K., Friedmann, E., Novac, N., Wels, W.S., Martoglio, B., Hueber, A.O. and Zornig, M. (2007) The Fas ligand intracellular domain is released by ADAM10 and SPPL2a cleavage in T-cells. Cell Death. Differ. 14, 1678–1687.
[6] Friedmann, E., Lemberg, M.K., Weihofen, A., Dev, K.K., Dengler, U., Rovelli, G. and Martoglio, B. (2004) Consensus analysis of signal peptide peptidase and homologous human aspartic proteases reveals opposite topology of catalytic domains compared with presenilins. J. Biol. Chem. 279, 50790–50798.
[7] Schröder, B., Wrocklage, C., Pan, C., Jäger, R., Kösters, B., Schafer, H., Elsässer, H.P., Mann, M. and Hasilik, A. (2007) Integral and associated lysosomal membrane proteins. Traffic 8, 1676–1686.
[8] Sannerud, R. and Annaert, W. (2009) Trafficking, a key player in regulated intramembrane proteolysis. Semin. Cell Dev. Biol. 20, 183–190.
[9] Bonifacino, J.S. and Traub, L.M. (2003) Signals for sorting of transmembrane proteins to endosomes and lysosomes. Annu. Rev. Biochem. 72, 395–447.
[10] Radons, J., Faber, V., Buhrmester, H., Volker, W., Horejsi, V. and Hasilik, A. (1992) Stimulation of the biosynthesis of lactosamine repeats in glycoproteins in differentiating U937 cells and its suppression in the presence of NH4Cl. Eur. J. Cell Biol. 57, 184–192.
[11] Koch-Nolte, F., Glowacki, G., Bannas, P., Braasch, F., Dubberke, G., Ortolan, E., Funaro, A., Malavasi, F. and Haag, F. (2005) Use of genetic immunization to raise antibodies recognizing toxin-related cell surface ADP-ribosyltransferases in native conformation. Cell Immunol. 236, 66–71.
[12] Schröder, B., Wrocklage, C., Hasilik, A. and Saftig, P. (2010) Molecular characterisation of 'transmembrane protein 192' (TMEM192), a novel protein of the lysosomal membrane. Biol. Chem. 391, 695–704.
[13] Behnke, J., Eskelinen, E.L., Saftig, P. and Schroder, B. (2011) Two dileucine motifs mediate late endosomal/lysosomal targeting of transmembrane protein 192 (TMEM192) and a C-terminal cysteine residue is responsible for disulfide bond formation in TMEM192 homodimers. Biochem. J. 434, 219–231.
[14] Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.
[15] Braulke, T. and Bonifacino, J.S. (2009) Sorting of lysosomal proteins. Biochim. Biophys. Acta 1793, 605–614.
[16] Lippincott-Schwartz, J. and Fambrough, D.M. (1987) Cycling of the integral membrane glycoprotein, LEP100, between plasma membrane and lysosomes: kinetic and morphological analysis. Cell 49, 669–677.